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Hepatitis C virus genotype 1b chimeric replicon containing genotype 3 NS5A domain

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Abstract

Infections with hepatitis C virus (HCV) genotype 3 exhibit differences in clinical phenotype including an increase in response to interferon therapy and development of steatosis. To initiate studies on genotype 3, we created a chimeric genotype 1b replicon containing a genotype 3a NS5A domain. The chimera was capable of efficient colony formation after the selection of a novel dominant adaptive mutation. Thus, domains from highly different strains can interact to form a functional replicase. A new genotype 1a replicon was constructed as well. Genotype specific influence on interferon sensitivity was examined using genotype 1a, 1b and chimeric 1b–3a replicons. The genotype 3a NS5A domain did not increase the sensitivity of the chimeric replicon to IFNα. The results suggest that NS5A is not sufficient to convey the increased IFNα response by genotype 3 or the replicon model is not capable of mimicking the events involved in increased sustained viral response.
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Keywords: HCV; Interferon stimulated genes; ISG; IFNα

Introduction

Hepatitis C virus (HCV) infections are one of the leading causes of liver disease worldwide with a prevalence of 2–3%. Infections are typically asymptomatic for decades, yet 20% of persistently infected individuals will eventually develop serious liver disease including cirrhosis and liver cancer. HCV infection is the leading cause for liver transplantation (Thomas and Seeff, 2005; Alter and Seeff, 2005), and liver cancer due to HCV infection is one of the most rapidly increasing types of cancer in the U.S. (Kim et al., 2005; El Serag, 2004). The current therapeutic treatment for HCV infection is pegylated IFNα2 and ribavirin (reviewed in (Feld and Hoofnagle, 2005) with rates of sustained viral clearance of 40–50% and 80–90% for genotype 1 and genotype 2/3, respectively (Manns et al., 2001; Fried et al., 2002).

Numerous studies have suggested that HCV evades the host IFN response by several mechanisms (reviewed in Gale and Foy, 2005). Enomoto et al. first described a relationship between resistance to IFNα therapy and sequence variation in a region of NS5A designated the interferon sensitivity determining region (ISDR) (Enomoto et al., 1996). Gale and colleagues demonstrated that this region interacts with PKR, providing a plausible mechanism for the modulation of the host response to IFN (Gale et al., 1997, 1998). The use of microarray analyses has supported the role of NS5A in blocking the IFN response (Geiss et al., 2003; Girard et al., 2002), yet these studies, as well as others, have suggested that PKR independent mechanisms may also be involved in the NS5A suppression of the IFN response (Podevin et al., 2001; Tan and Katze, 2001). Some studies have suggested that NS5A induces IL8 synthesis that in turn contributes to IFN resistance (Polyak et al., 2001; Girard et al., 2002). NS5A has been shown to interact with a number of proteins, including grb2, PI3 kinase (Tan et al., 1999) and hVAP (Evans et al., 2004; Tu et al., 1999).

Recently, HCV NS3/4A protease has been shown to block phosphorylation of IRF3 providing new insight into the mechanisms by which HCV subverts the dsRNA-induced IFN signaling pathways (Foy et al., 2003). NS3/4A blocks IRF3

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activation through both the RIG-I and TLR3 pathways. RIG-I is the dsRNA binding helicase that activates the IRF3 and NFκB pathways resulting in the induction of type 1 IFN (Yoneyama et al., 2004). The HCV NS3/4a protease blocks activation of IRF3 by this pathway (Foy et al., 2003, 2005) via cleavage of a newly identified adapter protein (CARDIF, IPS-1, MAVS, VISA) (Xu et al., 2005; Seth et al., 2005; Kawai et al., 2005; Meylan et al., 2005). TLR3 activation of IRF3 is suppressed by NS3/4A as well, via cleavage of its essential adapter protein TRIF (Li et al., 2005; Foy et al., 2003).

Although HCV does not replicate in conventional tissue culture systems, a surrogate system has been created based on a bicistronic replicon (Lohmann et al., 1999). The utility of this system was enhanced by the recognition that adaptive mutations arise that permit more efficient replication (Blight et al., 2000) and by the isolation of highly permissive cell lines derived by curing cells of the replicon (Blight et al., 2003; Yi and Lemon, 2004). The identification of adaptive mutations permitted the construction of replication competent full-length genomes without foreign sequences (Ikeda et al., 2002; Pietschmann et al., 2002). Recently, a true tissue culture system was realized for HCV in which infection of highly permissive cured Huh7 cell lines with a genotype 2 isolate results in efficient spread of the infection and secretion of infectious virions (Lindenbach et al., 2005, 2006; Wakita et al., 2005; Zhong et al., 2005). A genotype 1a clone capable of producing infectious virus has been described recently as well (Yi et al., 2006).

Infections with HCV genotype 3 are associated with an increase in sustained response to IFNα therapy (Manns et al., 2001; Fried et al., 2002) and an increase in risk for steatosis (Gochee et al., 2003; Monto et al., 2002; Kumar et al., 2002; Poynard et al., 2003). Currently, no genotype 3 replicon is available to address the mechanism for genotype specific differences in clinical outcome. To evaluate the role of NS5A in genotype specific differences in IFNα sensitivity, we have constructed a chimeric replicon containing the genotype 3a NS5A domain in a genotype 1b background. Adaptive mutations in NS3 and NS5A were required for replicon induced colony formation. The genotype 3a insert represents the simultaneous introduction of 92 amino acid changes into the genotype 1b background, demonstrating the remarkable capacity of the NS5A region for variation without loss of function. The genotype 3a NS5A domain did not increase the IFNα sensitivity of the 1b replicon, suggesting that either NS5A is not sufficient for the clinical phenotype of genotype 3 IFN sensitivity or that the current assays are not capable of mimicking the genotype 3 phenotype observed in the clinic.

Results

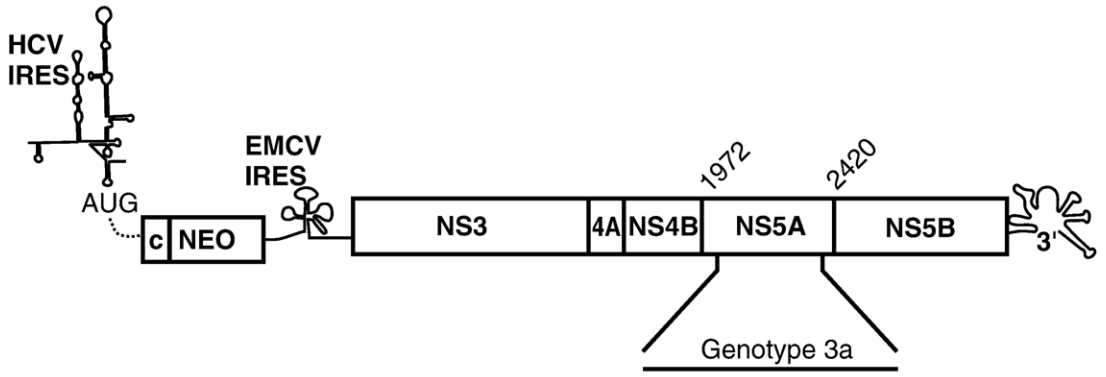
Selection of genotype 1b/3a chimeric replicons

To initiate studies on the genotype 3 specific clinical phenotypes, we created a series of chimeric replicons replacing the NS3–NS5B, NS5A–NS5B, and NS5A regions of a genotype 1b replicon (Rep1b) with genotype 3a sequences. NS5A was selected as the primary target for the initial studies, since it has been shown to correlate with response to exogenous IFNα in the clinic, and thus may be responsible for the genotype 3a phenotype. Although NS3/4A interferes with the endogenous production of type 1 IFN, it is not known to affect the response to exogenous IFNα. Modified versions of each replicon were constructed to contain the NS3-1431 mutation that enhances NS5A adaptive mutations in both the genotype 1a (see below) and 1b (Lanford et al., 2003) backgrounds. The only chimeric replicon capable of inducing colony formation was the replicon containing NS5A (hereafter designated Rep1b/3a). The NS5A genotype 3a insert spanned amino acid residues 2056 to 2406 and was 26% divergent from the genotype 1b sequence (92 of the 350 residues differed between genotype 3a and 1b; Fig. 1). Although transfection of parental Huh7 cells with Rep1b/3a without the NS3-1431 mutation failed to produce colonies on multiple attempts, transfection of a cell line cured of the genotype 1b replicon (Huh7-1bC) yielded G418 resistant colonies at a low level. In contrast, Rep1b/3a containing the NS3-1431 mutation was capable of low level colony formation on parental Huh7 cells and formed numerous colonies on the Huh7-1bC cell line (upper panel, Table 1). Rep1b/3a with or without the 1431 mutation failed to produce colonies in a Huh7 line cured of the genotype 1a replicon (Huh7-1aC) that was highly efficient for colony formation with a genotype 1a replicon (see below).

Cell lines were established from colonies derived by transfection of Rep1b/3a with and without the NS3-1431 adaptive mutation. The NS3 and NS5A domains were sequenced from seven cell lines (4 from Rep1b/3a and 3 from Rep1b/3a-1431). A dominant NS5A adaptive mutation was identified in 6 of 7 replicons at amino acid residue 2195 (proline to serine or threonine) (Table 2). The one replicon lacking a mutation at 2195 acquired a mutation at 2187 (alanine to glutamic acid) which has been identified as one of the residues involved in hVAP interactions (Evans et al., 2004) (see Discussion). The 2195 mutation has not been previously identified in genotype 1b replicons, but 2195 is within the domain of adaptive mutations identified in genotype 1b replicons that are located immediately upstream of the PKR binding and ISDR regions (Fig. 1B). NS3 mutations were acquired by all replicons not already possessing NS3-1431. Two of the replicons acquired NS3 mutations at 1109, which represents the most amino terminal NS3 mutation identified in any genotype 1b replicon to date. Thus, the inclusion of the genotype 3 NS5A domain resulted in the selection of adaptive mutations not previously recognized in genotype 1b replicons, suggesting that insertion of a divergent NS5A sequence can induce genotype specific compensatory mutations in distant domains of the polyprotein.

To test the effect of the NS5A-2195 mutation on colony formation, the P2195S mutation was introduced into Rep1b/3a with or without the NS3-1431 mutation. Direct comparison of the highly adapted Rep1b-1431/2204 (genotype 1b replicon

A



B

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1972 C S G S W L R D V W D W I C T V L T D F K T W L Q S K L L P R L P G V P F F S C Q R G Y K G V W R G D G I M Q T T C P C  1b
1972 . . . . . I . . . . E . . S . . . . . K A . . M . Q . . . I . . V . . . . . . . . . . . . . . . H . R . H .  1a
1972 . . D D . . . I I . . . V . S . . . . . . . S A . I M . A . . . L . . I . . . K . . . . . . . . . V . S . R . . .  3a

2032 G A Q I T G H V K N G S M R I V G P R T C S N T W H G T F P I N A Y T T G P C T P S P A P N Y S R A L W R V A A E E Y V  1b
2032 . . E . . . . . . . . T . . . . . . . . . R . M . S . . . . . . . . . . . . . . . L . . . . . T F . . . . . S . . . . .  1a
2032 . . T . . . . . . . . . . . L A . . . . . A . M . . . . . . . . . . . . . . S . . C . S . . . T . . . . . . . . N S . .  3a

2092 E V T R V G D F H Y V T G M T T D N V K C P C Q V P A P E F F T E V D G V R L H R Y A P A C K P L L R E E V T F L V G L  1b
2092 . I R Q . . . . . L . . . . . S . . . . . L . . . . . F . . P . . . . . . . . S . R . . .  1a
2092 . . R . . . . . . I . . A . E . E L . . . . . A . . . . . . . . . . . . . . . . P . . . . . D . I . . T A . .  3a

                                                                                    2195
                                                                                     *
2152 N Q Y L V G S Q L P C E P E P D V A V L T S M L T D P S H I T A E T A K R R L A R G S P P S L A S S S A S Q L S A P S L  1b
2152 H E . P . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . G . . . . . . . . . . V . . . . . . . . . . . . .  1a
2152 H S . T I . . . . . . . . . . . . . . . S . . . . . . R . . . . . . . . . . . A . . . . . . . . . . E . . . . . . . . . . . . . .  3a
                                                                                    2205 2209
                2163                                Adaptive Mutations

2212 K A T C T T R H D S P D A D L I E A N L L W R Q E M G G N I T R V E S E N K V V I L D S F E P L Q A E E D E R E V S V P  1b
2212 . . . . . A N . . . . . . E . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . D . . V . . . . . . . I . . .  1a
2212 . . . . Q . H R P H . . . E . V D . . . . . . . . . . S . . . . . . . . T . . . . . . . . . . . . R . . P . D A . L . . A  3a
                ISDR                                                2248

2272 A E I L R R S R K F P R A M P I W A R P D Y N P P L L E S W K D P D Y V P P V V H G C P L P P A K A P P I P P P R R K R  1b
2272 . . . . . K . . R . A Q . L . V . . . . . . . . . . . V . T . . K . . . E . . . . . . . . . . . . P . S . . V . . . . K . .  1a
2272 . . C F K K L P E Y . P . L . . . . . . . . . . . . . D R . . S . . . . . . T . . . . A . . . R S V . . V . . . . . . .  3a

2332 T V V L S E S T V S S A L A E L A T K T F G S S - - - - - E S S A V D S G T A T A S P D Q P S D D G D A G S D V E S Y S  1b
2332 . . . . T . . . L . T . . . . . . I . S . . . . . . . . . S T . G I T G D N T . T . S E P A P S G C P R D . . A . . . .  1a
2332 . I Q . D N . N . . A . . . A . . E R . . P . . K P Q E E D . . S S G V D . Q S G A T S K V P P S P G E E . . S . . C .  3a

2387 S M P P L E G E P G D P D L S D G S W S T V S E E A S - E D V V C C  1b
2387 . . . . . S . . . A . . . . . .  1a
2392 . . . . . C D . . . . . D S E E - Q S . . . .  3a
```

Table 1
Colony forming activity by chimeric genotype 1b/3a replicons

Replicon	Mutations		RNA Conc.	Huh7	Huh7-1aC	Huh7-1bC
	NS3	NS5A				
Rep1b/3a	–	–	10 μg	0	0	1
Rep1b/3a	1431	–	10 μg	3	0	112
Rep1b/3a	–	2195	100 ng			5
Rep1b/3a	1431	2195	100 ng			864
Rep1b	1431	2204	100 ng			1312

Cell lines were transfected with replicon RNA at the indicated levels (RNA Conc.) and colony formation was enumerated after selection with G418. The adaptive mutations present in NS3 and NS5A for each replicon are indicated. Huh7 cells were compared with cell lines cured of genotype 1a (Huh7-1aC) and 1b (Huh7-1bC) replicons. The upper and lower tables, divided by the horizontal line, represent different experiments, after and before the chimeric replicon was modified with the 2195 adaptive mutation.

containing NS3-1431 and NS5A-2204) (Lanford et al., 2003) and Rep1b/3a-1431/2195 (chimeric replicon with NS5A-2195 and NS3-1431) indicated that the chimeric replicon approached the same colony forming activity as the highly adapted Rep1b replicon (lower panel, Table 1). Thus, with the appropriate adaptive mutations, the highly divergent genotype 3a NS5A region did not significantly decrease colony formation in a genotype 1b background. These data demonstrate that regions of the polyprotein from highly divergent genotypes are capable of functional interaction for RNA replication.

Isolation of cell lines with genotype 1a replicon

To facilitate our studies on genotype specific IFN sensitivity, we constructed a genotype 1a replicon based on our infectious clone of the HCV-1 prototype strain (Lanford et al., 2001). In our previous investigation, this genotype 1a replicon failed to induce colony formation on parental Huh7 cells (Lanford et al., 2003) even after the addition of adaptive mutations at NS3-1431 and NS5A-2204 (Rep1a-1431/2204). However, following repeated large scale transfections, three G418 resistant colonies were produced on parental Huh7 cells, one of which produced a stable cell line. The HCV-1 strain is closely related to H77, the other genotype 1a replicon sequence, but differs by 78 amino acids of which 37 are in the NS3–NS5B domain. In contrast, HCV-1 is 12.3% divergent at the amino acid level from the genotype 1b Con1 strain within NS3–NS5B region (245 of 1987 residues differ; 49 in NS3 and 85 in NS5A; Fig. 1B). Sequencing of the NS3–NS5B coding region of the genotype 1a replicon revealed, in addition to the modifications at 1431 and 2204, a single mutation at NS3-1202 changing a glutamic acid to lysine. To test the contribution of the 1202 mutation to colony formation, it was inserted into Rep1a with and without the 1431 and 2204 mutations. Transfection of genotype 1a replicons with the different mutation combinations revealed that all three mutations were required for colony formation on parental Huh7 cells, but that either single NS3 mutation in combination with NS5A-2204 was sufficient to yield a low level of colonies on the cured cell lines Huh7-1aC and Huh7-1bC (Table 3). Since the Huh7-1aC line was derived from the original single genotype 1a colony from parental Huh7 cells, it was possible that this line represented the selection of cell type more permissive for the genotype 1a replicon; however, side by side comparisons revealed that the Huh7-1bC cell line was the most permissive for all Rep1a replicons, especially those with a single NS3 mutation (Table 3). Comparison of the Rep1a-1202/1431/2204 and Rep1b-1431/2204 replicons for colony formation following transfection at several RNA concentrations revealed that, although the Rep1a with 3 adaptive mutations was highly efficient at colony formation, it was less efficient than the highly adapted Rep1b-1431/2204 replicon (lower portion Table 3).

Steady state copy number for genotype 1a, 1b and chimeric 1b/3a replicons

The various replicon cell lines isolated during the course of these studies were compared for steady state copy number of the replicon. Cells were harvested under subconfluent growth conditions, and copy number was expressed as genome equivalents (GE) per μg of cell RNA (Fig. 2). On a per cell basis, steady state copy number ranged from 30 to 1300 GE per cell. All seven Rep1b/3a cell lines had lower copy number than either the Rep1b or Rep1a cell lines. On average, the Rep1b/3a lines had 4.5-fold lower copy number (Fig. 2). All the cell lines being compared were derived from the clonal Huh7-1bC cell line, so the cellular backgrounds were near identical in this comparison. The reduction in copy number in the Rep1b/3a lines appears to be characteristic of the chimeric replicon and may reflect a partial incompatibility of the genotype 3a NS5A sequence with the genotype 1b replicase that was not overcome by adaptive-compensatory mutations. However, since the entire genotype 3a NS5A sequence is not present in Rep1b/3a, the lower replication may be due to the chimerism of NS5A itself.

Genotype related sensitivity of replicons to IFNα

To examine the IFNα sensitivity of the chimeric genotype 1b/3a replicons in comparison to genotype 1a and 1b replicons,

Fig. 1. Schematic of the HCV replicon. (A) The bicistronic subgenomic HCV replicon is illustrated. The HCV IRES and EMCV IRES regulate translation of the neomycin phosphotransferase gene and the nonstructural proteins of HCV, respectively. The depiction of the HCV IRES was adapted from the structural studies of Honda et al. (1999). The nonstructural region of the polyprotein is depicted as a rectangle with demarcation of the individual viral protein domains. A domain of the NS5A protein is expanded to illustrate the region within NS5A of the Rep1b replaced with a genotype 3 sequence to create Rep1b/3a. (B) The sequence of the NS5A region is aligned for the comparison of the genotype 1a, 1b and 3a sequences. The amino acid numbering at the left is with respect to the HCV genome. The region containing clustered adaptive mutations is underlined (residues 2163–2205), as is the region containing the ISDR (2209–2248). The position of the dominant 2195 adaptive mutation arising in the Rep1b/3a replicon is indicated by an asterisk.

Table 2
Adaptive mutations in 1b/3a replicons

Replicon	NS3		NS5A	
Rep1b/3a Clone 22-1	1279	Thr→Lys	2195	Pro→Ser
			2203	Ala→Val
Rep1b/3a Clone 22-2	1109	Val→Asp	2195	Pro→Ser
	1202	Glu→Gly		
Rep1b/3a Clone 121-1	1226	Ala→Gly	2195	Pro→Thr
	1587	Val→Ile		
Rep1b/3a Clone 121-3	1279	Thr→Lys	2195	Pro→Thr
Rep1b/3a-1431 Clone 122-1	1431	Asp→Tyr	2195	Pro→Ser
Rep1b/3a-1431 Clone 122-4	1431	Asp→Tyr	2195	Pro→Thr
Rep1b/3a-1431 Clone 122-5	1109	Val→Asp	2187	Ala→Glu
	1431	Asp→Tyr		

Replicons resident in cell lines established with Rep1b/3a were sequenced as described in the text. Adaptive mutations in the NS3 and NS5A domains are indicated by amino acid number and amino acid change.

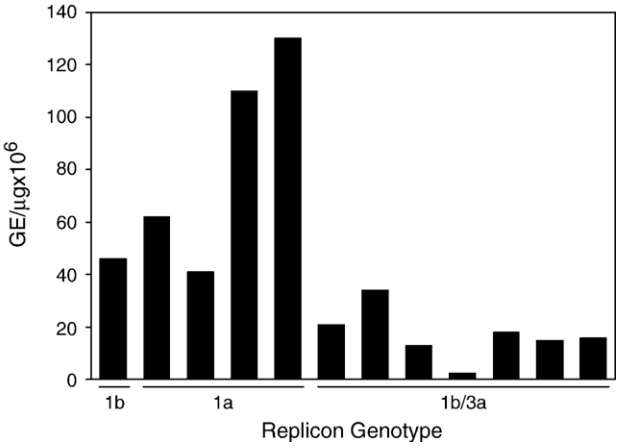

Fig. 2. Steady state copy number of the replicon RNA in cell lines. Cell lines containing various replicons were harvested under subconfluent growth conditions, and replicon RNA copy number was determined by TaqMan RT-PCR and expressed as genome equivalents (GE) per μg of cell RNA. All seven Rep1b/3a cell lines had lower copy number than either the Rep1b or Rep1a cell lines. All the cell lines being compared were derived from the clonal Huh7-1bC cell line, cured of the Rep1b replicon, so the cellular backgrounds were near identical in this comparison.

we utilized three approaches: (1) replicon response to high level IFNα; (2) replicon response to low level IFNα; and (3) host response to IFNα. In the first experiment, cell lines were treated with 1000 U/ml IFNα2b for 1, 2 or 3 days, and the replicon levels were determined by TaqMan RT-PCR. The level of replicon RNA declined by 4- to 10-fold by day 1, and 80- to 285-fold by day 3 (Fig. 3). The two Rep1b/3a cell lines did not exhibit a greater decline in replicon RNA levels than the Rep1a and Rep1b cell lines. The Rep1b and Rep1b/3a lines exhibited very similar responses to IFNα. Again, it should be noted that all of the replicon lines had the same cellular background, being derived from the clonal Huh7-1bC cell line. The greatest decline in replicon RNA was observed in the Rep1a Clone 4 line, which exhibited a 2-fold greater decrease in comparison to the other cell lines at all time points. Comparison of Rep1a Clone 4 to the other Rep1a cell lines suggested that this increased IFNα sensitivity was primarily due to clonal differences rather than a genotype difference (compare Rep1a-4 vs. Rep1a-8 and Rep1b-35, Fig. 4).

In the second set of experiments, we examined whether the Rep1b/3a replicons might be sensitive to lower levels of IFNα than the other replicons. Two cells lines for each replicon were treated in duplicate for 72 h with different levels of IFNα (0, 5, 25 and 100 U/ml). The Rep1b and Rep1b/3a lines were very similar and had IC_{50}s of approximately 5 U/ml, while the Rep1a

Table 3
Colony forming activity by genotype 1a replicons

Mutations				RNA Conc.	Colony count		
NS3			NS5A		Huh7	Huh7-1aC	Huh7-1bC
Rep1a	–	–	–	10 μg	0	0	0
	1202	–	–	10 μg	0	0	0
	1202	–	2204	10 μg	0	5	135
	–	1431	2204	10 μg	0	1	72
	1202	1431	2204	10 μg	704	~2000	~3000
Rep1b	–	1431	2204	0.01 μg	74	0	1056
	–	1431	2204	10 μg	nd	100	nd
Rep1a	1202	1431	2204	0.4 μg	nd	0	414
Rep1b	–	1431	2204	0.2 μg	nd	0	624

Cell lines were transfected with replicon RNA at the indicated levels (RNA Conc.) and colony formation was enumerated after selection with G418. The adaptive mutations present in NS3 and NS5A for each replicon are indicated. Huh7 cells were compared with cell lines cured of genotype 1a and 1b replicons. The upper and lower tables, divided by the horizontal line, represent different experiments. –, no mutation; nd, not done; TMTC, too many to count.

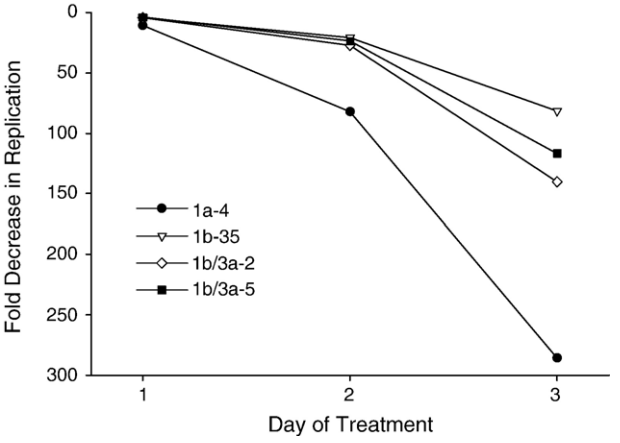

Fig. 3. Effect of replicon genotype on inhibition with IFNα treatment. Replicon cell lines derived with the Rep1a (clone 4), Rep1b (clone 35) and chimeric Rep1b/3a (clones 22-2 and 122-5) were treated with 1000 U/ml of IFNα for 3 days. Adaptive mutations present in the Rep1b/3a replicons are described in Table 2. Fresh medium and IFNα were provided on day 2. Replicon RNA levels were determined each day by quantitative RT-PCR and compared to untreated cells harvested at the same time to determine the fold decrease in RNA levels. RNA levels were first normalized as genome equivalents (GE) per μg of cell RNA. All cell lines were derived from the same clonal line (Huh7-1bC, cured of the Rep1b replicon) in order to have near identical cell backgrounds.

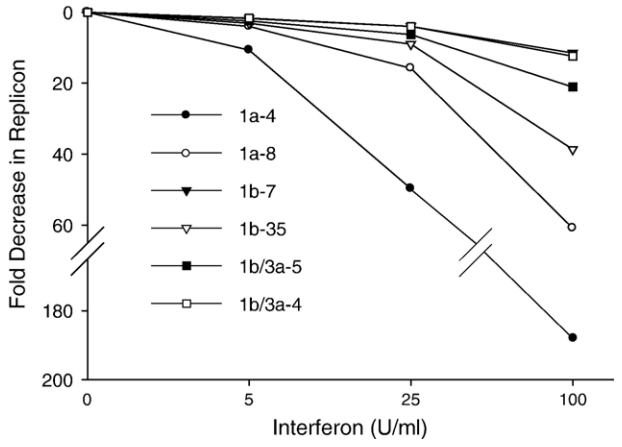

Fig. 4. Effect of genotype on IFNα IC_{50}. Duplicate cultures of cell lines derived with the Rep1a (clones 4 and 8), Rep1b (clones 7 and 35) and chimeric Rep1b/3a (clones 121-3 and 122-5) were treated with different levels of IFNα for 3 days. Adaptive mutations present in the Rep1b/3a replicons are described in Table 2. Fresh medium and IFNα were provided on day 2. Replicon RNA levels were determined each day by quantitative RT-PCR and compared to untreated cultures of each cell line harvested at the same time to determine the fold decrease in RNA levels. RNA levels were first normalized as genome equivalents (GE) per μg of cell RNA. With the exception of 1b-7, all cell lines were derived from the same clonal line (Huh7-1bC; cured of the Rep1b replicon) in order to have near identical cell backgrounds. The IFNα IC_{50} was approximately 5 U/ml for the 1b and 1b/3a cell lines and was less than 1 U/ml for the 1a cell lines.

lines had IC_{50}s of less than 1 U/ml. As above, the Rep1a cell lines exhibited marginally greater sensitivity to IFNα, and Rep1a Clone 4 was the most sensitive (Fig. 4). These data indicate that the genotype 3 NS5A region did not increase the IFNα sensitivity of the Rep1b replicon.

Finally, we examined the ability of the replicons to alter the downregulation of the ISG response. Our recent studies on host gene expression in uninfected chimpanzees following a single dose of IFNα revealed that the ISG response was rapidly downregulated in vivo in both the liver and PBMC. Most genes reached peak response within 4 h and had returned to baseline values by 24 h, even in the continuing presence of high levels of circulating pegylated IFNα (Lanford et al., 2006). Thus, the increased response of genotype 3a to IFNα therapy could potentially be related to a disruption in the ability of the cell to downregulate the ISG response. We selected six genes from the in vivo microarray data from chimpanzees to examine for the influence of the chimeric replicon on downregulation of the ISG response. We selected three genes that were rapidly downregulated in vivo and three genes that were not rapidly downregulated. The genes that were not rapidly downregulated by 24 h in vivo represented a small minority of the total ISGs (Lanford et al., 2006). The three representative genes that were rapidly downregulated in vivo included IFIT2 (alias ISG54), CXCL11 (alias I-TAC) and CXCL10 (alias IP-10). I-TAC and IP-10 represent CXCR3 chemokines that are expressed by hepatocytes (Bigger et al., 2004; Helbig et al., 2004; Lanford et al., 2006) and are involved in recruitment of lymphocytes to sites of inflammation. The three representative genes that were not downregulated in vivo included MX1, OAS3 and ISG12. MX1 and OAS are known to have antiviral activity in a number of model systems.

Huh7 cells, Huh7-1bC and three replicon lines (one each for genotypes 1a, 1b and 1b/3a) were treated with IFNα at 1000 U/ml for 72 h with changes of medium and IFNα every 24 h. Analysis of replicon levels revealed a pattern similar to those described above. A 24 h lag in the decline of replicon levels was followed by a decline of 95- to 143-fold over 72 h, with the chimeric replicon being no more sensitive to IFNα than the other genotypes (Table 4). The ISG transcripts were upregulated from 20-fold to greater than 2000-fold following IFNα treatment (Table 4). Transcript levels were measured by TaqMan RT-PCR at 4, 8, 24, 48 and 72 h. The representative genes that were rapidly downregulated in vivo were also rapidly downregulated in Huh7 cells, the cured cell line and all three of the replicon lines (Fig. 5). The genes not downregulated in vivo were also not downregulated in Huh7 cells or the replicon lines. None of the replicons appeared to

Table 4
Interferon induced increase in ISG transcripts in replicon cell lines

RNA	Time	Huh7	Huh7-1bC	Rep1b	Rep1a	Rep1b/3a
HCV	4 h			1	1	1
	8 h			1	1	1
	24 h			−4	−5	−4
	48 h			−36	−48	−21
	72 h			−143	−121	−95
IFIT2	4 h	691	603	534	317	578
	8 h	369	440	940	186	480
	24 h	7	9	52	22	104
	48 h	6	5	10	6	14
	72 h	6	7	13	11	2
CXCL11	4 h	11	21	8	50	12
	8 h	35	80	21	104	25
	24 h	3	3	20	23	9
	48 h	4	3	7	3	3
	72 h	3	Nd	nd	3	3
CXCL10	4 h	8	4	4	24	7
	8 h	23	18	47	14	13
	24 h	3	2	13	16	7
	48 h	6	3	1	3	3
	72 h	2	2	2	3	2
MX 1	4 h	112	338	241	96	106
	8 h	700	1828	2333	944	357
	24 h	500	800	919	371	409
	48 h	222	828	696	238	656
	72 h	126	559	415	131	465
OAS3	4 h	8	36	44	28	29
	8 h	29	155	200	93	236
	24 h	14	71	73	36	138
	48 h	15	75	79	28	106
	72 h	22	63	50	22	93
ISG12	4 h	14	12	16	12	9
	8 h	113	228	144	131	100
	24 h	114	231	141	143	152
	48 h	147	158	147	66	177
	72 h	223	270	175	91	219

Cell lines were treated with 1000 U/ml of IFNα for 72 h. Fresh media and IFNα were provided every 24 h. The levels of replicon RNA and ISG transcripts were determined by quantitative RT-PCR and expressed as fold change in comparison to untreated cultures harvested at each time point.

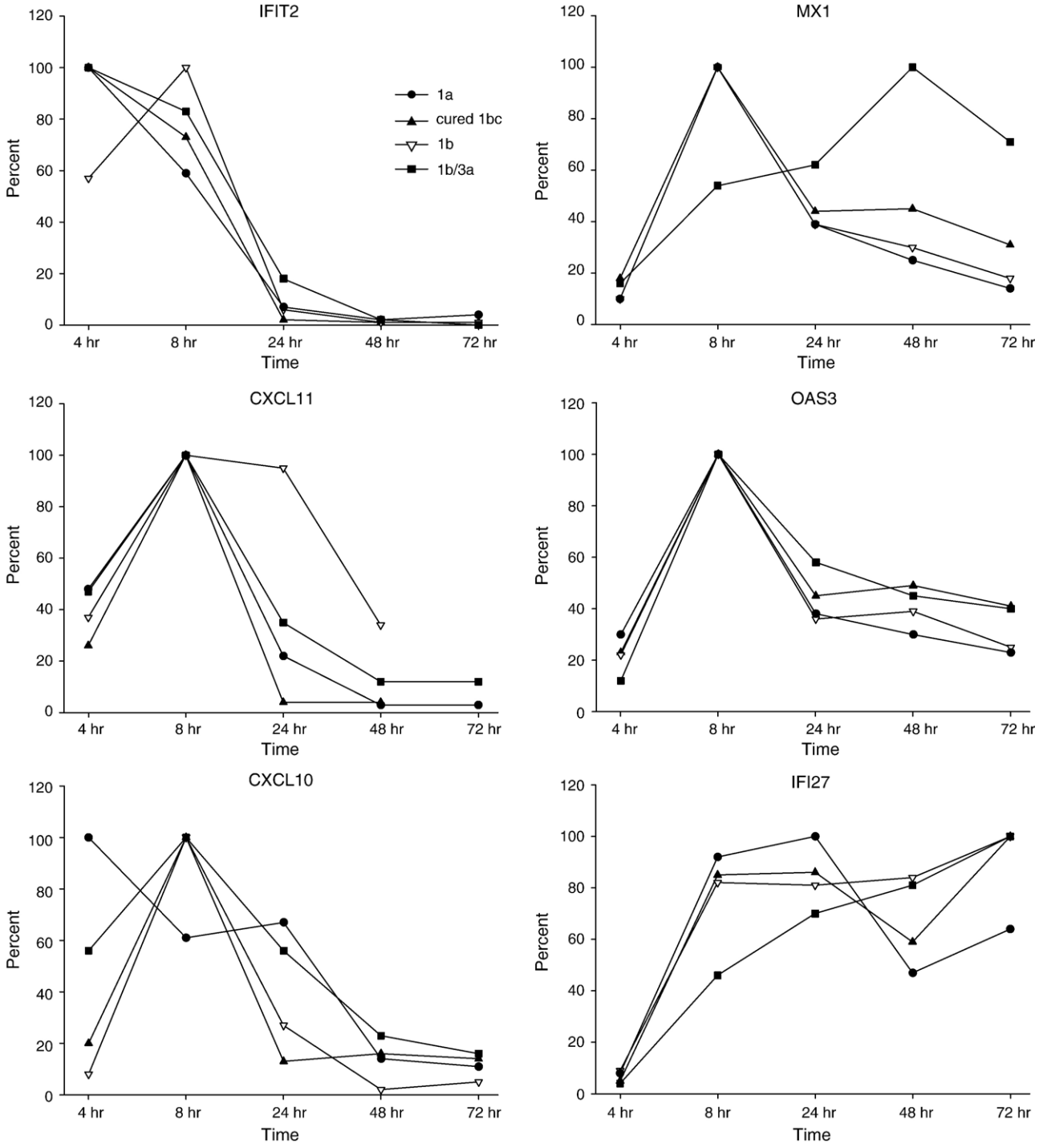

Fig. 5. Effect of replicon genotype on ISG induction upon IFNα treatment. Cell lines derived with Rep1a (clone 8), Rep1b (clone 35) and chimeric Rep1b/3a (clone 122-5) and the Huh7-1bC cured line were treated with IFNα for 72 h with fresh medium and IFNα being provided every 24 h. Treated and untreated cultures were harvested at 4, 8, 24, 48 and 72 h. Total cell RNA was analyzed by quantitative RT-PCR to determine the levels of transcripts for the interferon stimulated genes IFIT2 (ISG54), CXCL11 (I-TAC), CXCL10 (IP-10), MX1, OAS3 and IFI27 (ISG12). Fold increase was determined by normalization to transcript copy per μg of cell RNA and comparison to untreated cultures of each cell line harvested at the same times (see Table 4 for fold increase values). Percent increase was determined for each transcript by normalization to the highest value during the time course.

significantly alter the induction of these ISGs or their downregulation in comparison to the Huh7-1bC cell line used for their derivation (Table 4; Fig. 5). Thus, the NS5A genotype 3a insert did not alter the host response to IFNα or the ability of the cell to downregulate the ISG response.

Discussion

The development of a replicon system for HCV has provided a means to conduct numerous studies not previously possible, including the evaluation of antiviral compounds. Studies with

the replicon have demonstrated the direct antiviral activity of IFNα and IFNγ (Lanford et al., 2003; Frese et al., 2002; Blight et al., 2000; Guo et al., 2001; Frese et al., 2001; Cheney et al., 2002). In the current study, we developed a chimeric 1b/3a replicon containing the genotype 3a NS5A region and a new genotype 1a replicon based on the HCV-1 genotype 1a sequence. An important aspect of these studies was the development of cell lines cured of the replicon by antiviral treatment. Genotype 1a replicons were originally described by Blight et al. (2003). Similar to our original findings (Lanford et al., 2003), these investigators failed to obtain colony formation in Huh7 cells even with a 1a replicon containing the NS5A-2204 adaptive mutation. However, a low level of colony formation was attained by using a highly permissive Huh7 variant derived by curing a replicon line with IFNα (Blight et al., 2002). The established 1a replicons had acquired mutations in NS3 at 1226 and 1496. Gu et al. also selected a cell line with a 1a replicon based on the H77 sequence containing the 2204 mutation. The replicon had acquired mutations in NS3 at residues 1358 and 1609 (Gu et al., 2003). Yi et al. described a highly efficient H77 genotype 1a replicon derived by progressively replacing domains of the genotype 1b sequence with genotype 1a with sequential addition of selected adaptive mutations into the genotype 1a replicon (Yi and Lemon, 2004). Reconstruction of a full-length clone with this adapted H77 sequence provided high level replication and production of infectious particles in Huh7.5 cells (Yi et al., 2006).

The 1a replicon in the current study was based on an infectious clone (Lanford et al., 2001) of the prototype HCV-1 sequence and is the only non-H77 genotype 1a replicon described. The HCV-1 sequence was not capable of significant colony formation on Huh7 cells, despite adaptive mutations at both NS3-1431 and NS5A-2204. The acquisition of a second NS3 mutation at 1202 dramatically altered the host range and colony forming activity of this replicon. The 1a with two NS3 mutations was comparable in colony forming activity to the adapted 1b replicon on cells cured of the genotype 1b replicon (Huh7-1bC) and exceeded the 1b replicon on cells cured of the 1a replicon (Huh7-1aC).

The significance of NS3 mutations has not been elucidated, but in our studies, their primary effect appears to be the enhancement of NS5A mutations since by themselves NS3 mutations do not appear to enhance replicon activity (Lanford et al., 2003). Almost all of the NS3 adaptive mutations described to date in both genotype 1a and 1b replicons lie within residues 1202 to 1609 of the helicase domain. Interestingly, the 1b/3a replicons extended the border for genotype 1b NS3 mutations to include a mutation at 1109, well within the protease domain. Two independent lines contained this mutation, each of which had a second distinct NS3 mutation (1202 or 1431). The only other report of an adaptive mutation in the protease domain was an NS3 mutation at residue 1067 in a genotype 1a replicon (Yi and Lemon, 2004).

The isolation of replicons based on divergent genotypes has provided a means to explore the nature of genotype specific differences in IFNα sensitivity. We engineered replicons based on the 1b sequence with a genotype 3a NS5A domain since multiple studies have implicated NS5A in evasion of host IFN response. It was surprising that a chimeric containing an NS5A sequence with 26% divergence from the 1b NS5A (equivalent to 92 simultaneous amino acid substitutions) would be replication competent. NS5A has been shown to be highly tolerant of mutations in the replicon model. The initial studies on adaptive mutations revealed a deletion of 47 amino acids downstream of the hyperphosphorylation domain that enhanced replication (Blight et al., 2000). Another deletion was identified in a replicon recovered from HeLa cells (Zhu et al., 2003). Additional studies have revealed that the carboxyl terminus of NS5A will accept large insertions including GFP (Fiedler et al., 2004; Moradpour et al., 2004). Yet, random insertions throughout the NS5A domain were lethal except for two positions in the C-terminus of the protein (Moradpour et al., 2004). Collectively, these studies indicate that the replicon system is tolerant of changes in specific regions of NS5A and at the same time is highly dependent on specific adaptive mutations in NS5A.

NS5A is a zinc-binding protein of unknown function (Tellinghuisen et al., 2004) that exists as two phosphorylated forms: basal phosphorylated p56 and hyperphosphorylated p58. Adaptive mutations within and outside of NS5A tend to decrease hyperphosphorylation (Evans et al., 2004; Appel et al., 2005; Blight et al., 2000), and thus an association exists between decreased phosphorylation and RNA replication. This concept is further supported by the effect of kinase inhibitors that decrease NS5A p58 and increase HCV RNA replication (Neddermann et al., 2004). The association extends further to include the interaction with hVAP. Mutations affecting hyperphosphorylation increase the interaction between hVAP and NS5A, including adaptive mutations in NS4B (Evans et al., 2004). Interestingly, the dominant adaptive mutation in the genotype 1b/3a replicon was a proline to serine/threonine mutation and thus differs from most adaptive mutations in being the addition of a potential phosphorylation site rather than the loss of a serine or threonine. Nonetheless, presumably the 2195 mutation functions for genotype 3 NS5A in a manner similar to the 2204 mutation for genotypes 1a and 1b. The one clone that did not contain the adaptive mutation at 2195 contained a mutation at 2187, one of the residues involved in hVAP interaction.

The ISDR of NS5A (Enomoto et al., 1996) has been recognized in multiple clinical studies, and in vitro studies of variant NS5A proteins have supported the importance of this region in IFNα response. Different studies have supported both PKR-dependent (Gale et al., 1997, 1998) and PKR-independent modulation of the IFNα response by NS5A (Geiss et al., 2003; Girard et al., 2002; Podevin et al., 2001; Tan and Katze, 2001). Amino acid variations in genotype 3 strains that were associated with IFNα resistance were independent of the PKR pathway (Castelain et al., 2002). Our studies did not observe an increased sensitivity to IFNα for 1b replicons containing a genotype 3a NS5A. One possible interpretation of these results is that the NS5A region is not sufficient to convey the genotype 3 phenotype for increased IFNα sensitivity. Recent studies have demonstrated that NS3/4a blocks the dsRNA-induced activation

of IRF3 (Foy et al., 2003; Li et al., 2005; Foy et al., 2005), a critical component of the type 1 IFN response pathway. Although NS3/4a is involved in blocking endogenous type 1 IFN production, no data suggest that it is involved in resistance to exogenous sources of IFN. Other viral proteins may be involved in HCV resistance to IFN, as well. However, it is not clear that the replicon system is the appropriate model to test for this phenotype. Increased sustained viral clearance is a complex end point that is not completely understood and may not necessarily translate to an observable in vitro phenotype. Full-length genotype 3 replicons without foreign sequences may be required to determine whether the genotype 3 phenotype of increased sustained viral response in patients can be observed as an in vitro phenotype.

Materials and methods

Replicon constructs

Rep1b was previously described (Lanford et al., 2003) and was constructed to be an exact copy of the replicon described by Lohmann et al. (1999) (GenBank accession no. AJ242652). A highly adapted version of this genotype 1b replicon containing the NS3-1431 and NS5A-2204 mutations (Rep1b-1431/2204) was isolated and characterized in our previous studies (Lanford et al., 2003). The chimeric Rep1b/3a replicons were constructed by inserting the NS5A coding region from a genotype 3a strain into the Rep1b backbone (Figs. 1A and B). The HCV genotype 3a strain was recovered from a chronically infected chimpanzee that was originally inoculated with a NonA, NonB hepatitis contaminated blood product. The NS5A region was amplified from serum derived RNA using primers that spanned nucleotide numbers 5041 to 6041 (GenBank; AY587021), and thus the amino and carboxy termini of NS5A (93 and 19 amino acids, respectively) are still genotype 1b in the chimeric replicon. The forward and reverse primers included restriction sites for *Mlu*1 and *Bam*H1, respectively, which are unique in the Rep1b construct and allowed for the insertion of the genotype 3a sequences in-frame with the Rep1b sequences. The Rep1b/3a replicons were further modified by introduction of the NS3-1431 mutation that is highly synergistic with NS5A adaptive mutations (Lanford et al., 2003) and NS5A-2195 that was a dominant mutation selected after transfection of Rep1b/3a. The Rep1a construct has been previously described (Lanford et al., 2003) and is identical to the Rep1b construct except that all HCV components were derived from the HCV-1 genotype 1a infectious clone (Lanford et al., 2001) (GenBank accession no. AF271632) including the 5′ NCR, the NS3–NS5B encoding region and the 3′ NCR. The Rep1a variant used in this study contained 228 nucleotides (1927 to 2155) derived from Rep1b, which altered 12 of the first 73 amino acids of NS3 (Lanford et al., 2003). Subsequent replacement of this region with the 1a sequence did not noticeably affect the activity of the replicon.

Adaptive mutations in the Rep1a cell lines were identified by direct sequencing of PCR products that spanned the entire NS3-5 open reading frame, while adaptive mutations in the Rep1b/3a cell lines were detected by direct sequencing of PCR products that spanned the entire NS3 domain (3420–5474) and most of the NS5A domain (6527–7474). Specific adaptive mutations were introduced into replicons using PCR directed mutagenesis. Synthetic replicon RNA was prepared from *Sca*1 (Rep1b and Reb1b/3a) and *Xba*1 (Rep1a) linearized DNA using the T7 Megascript Kit (Ambion, Austin, TX) and was purified by DNase treatment, RNazol (Leedo; Houston, TX) extraction and ethanol precipitation. RNA was quantified by optical density, and concentrations and quality were confirmed by agarose gel electrophoresis.

Cells and transfections

Huh7 cells were cultivated in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml of gentamicin sulfate. RNA transfections were performed using DMRIE-C (Carlsbad, CA). Replicons with low or no colony forming efficiency were transfected using 10 μg of replicon RNA per 100 mm culture dish; however, replicons with adaptive mutations were transfected at 1 to 0.01 μg of replicon RNA adjusted to 10 μg with Huh7 RNA. Culture medium was supplemented with 250 μg/ml of G418 beginning 1 day after transfection. This transfection protocol routinely yielded 25–50% transfection efficiency for RNA as evaluated by transfection of a Sindbis replicon expressing β-galactosidase. Cell lines highly permissive in replicon colony formation assays were derived by using IFNα to cure cells of the genotype 1b (Huh7-1bC) or genotype 1a (Huh7-1aC) replicons, as previously described (Blight et al., 2002).

TaqMan quantitative RT-PCR for replicon RNA and host transcripts

Total cell RNA was isolated from cell cultures using RNazol (Leedo, Houston, TX). Replicon RNA was quantified by a real time, 5′ exonuclease RT-PCR (TaqMan) assay exactly as previously described (Lanford et al., 2001, 2003). The conditions for quantification of transcripts from IFN-stimulated genes (ISGs) were identical to those described for replicon RNA. Assays for host transcripts were purchased from ABI as Assays on Demand (Foster City, CA).

Antiviral treatments

IFNα2b (Intron A) was a gift from Schering Plough Research Institute (Kenilworth, NJ). Antiviral studies were initiated 24 h post-plating when cultures were still subconfluent. None of the antiviral treatments induced noticeable toxicity by microscopic inspection of the cultures.

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